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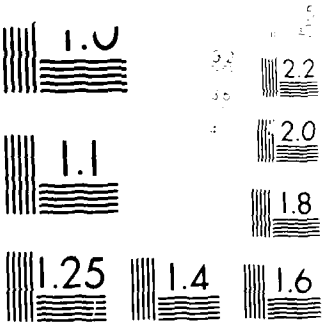
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Work is currently in progress on establishing a consistently reproducible paradigm for stress-induced immunosuppression in the mouse. Preliminary data indicate decreases in spleen and thymus weights, and in T-cell responsivity to mitogens following brief periods of restraint or electric footshock, especially with repeated treatments. Other preliminary data indicate that brain dopaminergic and noradrenergic systems respond acutely to the injection of a viral antigen (NDV), in addition to the previously reported increases in plasma corticosterone. The cerebral responses are particularly evident in hypophysectomized mice, in which we do not observe substantial increases in plasma corticosterone. We have not observed consistent changes in cerebral amines following injection of activated spleen cell supernatants, or thymosin fraction V (Tf-5). In a collaborative study, we found that Tf-5 does not directly alter corticosterone binding to its brain receptors, thus ruling out one potential mechanism for the anti-stress properties of Tf-5.

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In accordance with our original proposal, our major initial efforts have been directed towards the task of establishing a consistently reproducible paradigm for the production of stress-related deficits in immune function. To this end, several experiments have been performed using various stressors in the male CD-1 mouse. The stressors we have employed to date are electric footshock and restraint, two procedures used commonly in the field of research on stress. The advantages of these two procedures are that the degree of stress is readily manipulable by, for example, altering the intensity of the footshock, or the duration of the period of stress. The emphasis has been to determine the minimum stressful treatments that will produce consistent results, both for humane reasons, and for reasons of experimental expedience. We have typically used 20-40 footshocks over periods of 15 to 30 minutes, using shock pulses of 1 second duration and a current of 0.2 mA, or restraint for 30 to 120 minutes. We have also examined the relative effects of acute (one session) treatments, and chronic (repeated two to seven times) treatments.

For these initial studies we have restricted the number of immune measures, measuring only thymus and spleen weights, spleen cell counts, and the mitogen responsivity of splenic cells. In the forthcoming year we plan to add an assay for interleukin II (IL-2) production to these screening procedures. Moreover, we have in all cases measured plasma corticosterone, and in most cases, the biogenic amines and catabolites in the prefrontal cortex, hypothalamus, and brain stem of the mice.

The results obtained on the cerebral amines are entirely consistent with our earlier results (see Fig. 1). The analyses are performed directly on perchloric acid extracts of the brain tissue by high performance liquid chromatography using electrochemical detection (HPLC-EC) as described in the original application. The principal metabolites of interest are dihydroxyphenylacetic acid (DOPAC), the major catabolite of dopamine (DA), 3-methoxy,4-hydroxyphenylethyleneglycol (MHPG), the major catabolite of norepinephrine (NE), and 5-hydroxyindoleacetic acid (5-HIAA), the major catabolite of serotonin (5-HT). There is reason to believe that DOPAC:DA, MHPG:NE, and 5-HIAA:5-HT ratios reflect activity in DA, NE and 5-HT neurons respectively. Our work (Dunn, 1986) replicating that of others, indicates widespread increases of MHPG:NE in all brain regions studied following any stressor. In addition, there is a sensitive increase of DOPAC:DA in the prefrontal cortex, and to a lesser extent in other regions of the brain, including the hypothalamus and brain stem. 5-HIAA:5-HT is increased in several regions with the more intense stressors. Our work in mice has not revealed any dramatic differences in these responses to the different stressors we have studied so far.

The principal results so far from the immunological analyses have indicated consistent reductions in spleen and thymus weights, particularly in the repeated or chronically stressed animals (see example, Table I). Mitogen responsivity is also decreased, particularly using the T-cell mitogen, phytohemaglutinin (PHA), but not the B-cell mitogen, lipopolysaccharide (LPS) (see example, Table II).

However, in the course of these experiments, we have experienced considerable variability in both cell viability and mitogen responsivity. Our preliminary experiments have established two principal causes for this problem. When we conducted pilot experiments on the feasibility of freezing lymphocytes

and shipping them to Washington for analysis, cell viability on thawing was very high, much higher than we have achieved in our stress experiments. The major reason for this was the delay involved in processing the tissue prior to freezing. When only a few animals are involved, this delay can be very short and cell viability is high. Our stress experiments must involve at least 16 animals for a minimum "n" of 8, and frequently we use considerably more animals than this. This increases the time delay in processing and the cell viability and mitogen responsivity suffers. We determined early on that it was important to disperse the spleen cells very soon after excision, rather than to save the cells and process them together which would be more convenient, and is acceptable for the neurochemical samples. Our most recent studies indicate that if the spleen cells are dispersed immediately after excision, and the cells are processed in small batches, excellent viability and mitogen responsivity are retained (see Table II). We believe that it is important to continue our studies on these problems involved with the shipping of cells, even though the potential exists to set up many of these procedures at the University of Florida. This is because of the potential importance of this procedure for research in neuroimmunology, which, because of the diverse nature of the techniques employed, frequently necessitates collaborative efforts between separate laboratories.

In another line of research, we have been investigating whether or not the CNS responds to the injection of virus, which might be regarded as a potential stressor. For this we have adopted the model of Smith et al. (1982) using Newcastle Disease virus (NDV). NDV is not infectious in the mouse, and probably acts merely as an antigenic stimulus. We have been able to verify that there is a delayed increase in plasma concentrations of corticosterone with a peak at about 8 hours following injection as Smith et al. had reported (Table III). Based on the results of three experiments, we have not observed substantial increases following virus injection in the plasma corticosterone of hypophysectomized mice that had been verified for the completeness of the hypophysectomy, by testing the plasma corticosterone response to restraint stress or CRF administration. This is a vital control procedure (not performed by Smith et al.) because it is well established that "hypophysectomized" rodents may retain some pituitary-adrenal function if any tissue is left in the sella tursica, even though this may not be detected by visual inspection (Moldow and Yalow, 1979). These results have been submitted to cience for publication as a Technical Comment on the paper of Smith et al. (1982).

In these experiments, we have, however, repeatedly observed alterations in the cerebral biogenic amine metabolism consistent with an activation of cerebral dopaminergic, and perhaps also noradrenergic systems, especially in hypophysectomized mice (Table IV). These preliminary results are potentially important, because they suggest that the brain does indeed respond acutely to an antigenic stimulus, so that it may be capable of coordinating a response.

In a separate series of experiments, we have investigated the ability of various thymic extracts to initiate cerebral biogenic amine responses, specifically to verify the report of Besedovsky et al. (1983) that injection of a concanavallin-A (Con-A)-stimulated spleen cell supernatant decreases the hypothalamic content of norepinephrine. This decrease of hypothalamic NE was interpreted to indicate an activation of noradrenergic systems in this region. These systems have been implicated in the control of the release of corticotropin-releasing factor (CRF), which in turn stimulates ACTH, and hence corticosterone release (i.e. the classic activation of the hypothalamic-pituitary-adrenal axis (Selye, 1950)). In three separate

experiments, we have tested the effect of injections into mice of this same preparation or thymosin fraction 5 (Tf-5) on the cerebral content of biogenic amines and their catabolites. In no case have we observed results supportive of Besedovsky's report. A much more sensitive index of noradrenergic activation is the production of the catabolite, MHPG. We have not found this compound to be altered significantly in any of our experiments (see example, Table V), even though we have observed significant elevations of plasma corticosterone by both the lymphokine-containing preparation and Tf-5 (Table VI). These data are consistent with other experimental data indicating that these thymic extracts act at the level of the pituitary (McGillis, Hall and Goldstein, submitted).

In one other related study we have investigated one potential mechanism by which Tf-5 might reverse stress-induced immunosuppression. Previous studies reviewed in the original application suggested that Tf-5 can block the binding of dexamethasone to intact cells (lymphocytes). Thus, in collaboration with the laboratory of Dr. William Luttge at the University of Florida, which has extensive experience with glucocorticoid receptor assays, we investigated the ability of Tf-5 to compete for corticosterone binding to its receptors. Two experiments using partially purified cerebral glucocorticoid receptors showed absolutely no effect of Tf-5 on steroid binding (Fig. 2). Thus the ability of Tf-5 to counteract glucocorticoid effects cannot be explained by a direct action on glucocorticoid receptors. This result suggests that if indeed Tf-5 is capable of interfering with steroid binding it may occur at the level of the membrane, possibly by blocking the passage of the steroid into the cell. Other ongoing work in Dr. Hall's laboratory suggests that Tf-5 can modify Ca^{2+} flux across the membrane.

The work reported here represents the results obtained in only the initial nine months of this project, because although the official contract initiation date was April 1, 1985, funds were not received by the University of Florida until June, and these were not available to the investigators until late June.

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Table I Spleen and Thymus Weights

Treatment	Spleen Weight (mg)	Thymus Weight (mg)	Spleen Cell Count (x 106)
Quiet	130 ± 9	57 ± 3	67 ± 10
Acute Restraint	111 ± 7+	55 ± 5	77 ± 11
Chronic Restraint	103 ± 5*	40 ± 6*	63 ± 8

Acute restraint was for 1 hr once; chronic restraint was for 1 hr per day on seven successive days. Mice were sacrificed immediately after the last period of restraint. *$P < 0.05$; +$0.1 > P > 0.05$ relative to quiet mice.

Table II Mitogen Responsivity of Spleen Cells of Restrained and Quiet Mice

Treatment	Media	LPS	LPS-SI	PHA	PHA-SI
Quiet	2150	38600	31.3	9700	5.8
	(730)	(6900)	(11.7)	(3000)	(1.1)
Restrained	2350	36900	31.9	5420*	3.1*
	(730)	(4500)	(10.0)	(1050)	(0.4)

Mice were restrained for two hours per day on four successive days and samples collected immediately after the last period of restraint. Mitogen responsivity to 5 ug of lipopolysaccharide (LPS) or 0.1% phytohemaglutinin (PHA) were tested at 48 hr. Numbers are the mean (SEM) cpm of accumulated [^{3}H]. The fourth and sixth columns are the respective stimulation indices for LPS and PHA (i.e. stimulated/media).

*$P < 0.05$ relative to quiet mice.

Table III Changes in Plasma Corticosterone following Injection of NDV

Treatment	Time after Injection (hours)	Plasma Corticosterone (ng/ml)
Saline	2.0	115 ± 35
Virus	2.0	340 ± 67*
Virus	4.0	234 ± 23*
Virus	8.0	955 ± 240*

300 ul of saline or Newcastle Disease Virus (NDV) containing 750 HA Units were injected IP into mice. Injections were staggered so that all plasma samples were collected at the same time of day. Plasma corticosterone was determined at various times after the injection by radiommunoassay.

*$P < 0.05$ relative to saline.

Table IV Cerebral Amines following Virus Injection in Sham and Hypophysectomized Mice

Brain Region	Amine	Sham Saline	Sham Virus	Hypox Saline	Hypox Virus
Frontal Cortex	NE	.62 ± .04	.53 ± .04	.62 ± .06	.52 ± .06
	MHPG	.18 ± .008	.179 ± .006	.145 ± .005	.2 ± .014
	MHPG/NE	.298 ± .023	.353 ± .031	.249 ± .034	.413 ± .058*
	DA	.051 ± .001	.05 ± .004	.05 ± .003	.078 ± .007*
	DOPAC	.034 ± .002	.036 ± .003	.045 ± .003*	.082 ± .011*
	DOPAC/DA	.67 ± .032	.707 ± .027	.913 ± .062	1.05 ± .078
Hypothalamus	NE	2.09 ± .119	1.97 ± .12	2.01 ± .15	1.55 ± .133*
	MHPG	.362 ± .019	.364 ± .012	.312 ± .015	.501 ± .021*
	MHPG/NE	.175 ± .009	.188 ± .009	.157 ± .009	.329 ± .016**
	DA	.7 ± .09	.68 ± .04	.71 ± .07	.99 ± .12
	DOPAC	.193 ± .015	.201 ± .008	.258 ± .025*	.365 ± .026*
	DOPAC/DA	.293 ± .025	.304 ± .02	.372 ± .039*	.387 ± .045
Brain Stem	NE	.757 ± .07	.8 ± .028	.914 ± .028	.811 ± .041
	MHPG	.134 ± .012	.148 ± .008	.132 ± .006	.233 ± .018
	MHPG/NE	.178 ± .008	.185 ± .008	.144 ± .004*	.287 ± .013**
	DA	.086 ± .009	.09 ± .005	.094 ± .006	.12 ± .012
	DOPAC	.048 ± .006	.058 ± .004	.072 ± .004*	.127 ± .014*
	DOPAC/DA	.555 ± .044	.645 ± .024*	.773 ± .059*	1.06 ± .09*

Sham-operated or hypophysectomized (hypox) mice were injected IP with 0.3 ml of Newcastle Disease Virus and samples collected 8 hours later. Amines were determined by HPLC-EC. Statistical comparisons were to the appropriate saline-injected group for virus-injected mice, and relative to sham-operated controls for hypophysectomized-saline mice. *$P < 0.05$, **$P < 0.001$.

Table V Cerebral Biogenic Amines following Thymosin Fraction 5 Injection

Tissue Content (ng/mg)

rain Region	Amine	Uninjected	Saline	Tf-5 (500 ug)	Tf-5 (1000 ug)
refrontal ortex	NE	.732 ± .043	.682 ± .047	.778 ± .039	.739 ± .033
	MHPG	.26 ± .011	.27 ± .012	.279 ± .013	.291 ± .017
	MHPG/NE	.375 ± .05	.408 ± .032	.344 ± .016	.399 ± .029
	DA	.089 ± .018	.065 ± .005	.071 ± .006	.066 ± .003
	DOPAC	.053 ± .007	.048 ± .004	.057 ± .006	.048 ± .004
	DOPAC/DA	.665 ± .078	.749 ± .062	.803 ± .061	.741 ± .055
eptum	NE	1.11 ± .04	1.23 ± .07	1.21 ± .09	1.14 ± .04
	MHPG	.382 ± .018	.366 ± .02	.41 ± .03	.421 ± .017
	MHPG/NE	.343 ± .013	.305 ± .027	.348 ± .03	.374 ± .02
	DA	3.03 ± .47	2.32 ± .38	3.1 ± .53	1.92 ± .29
	DOPAC	.596 ± .077	.575 ± .042	.735 ± .105	.487 ± .047
	DOPAC/DA	.207 ± .014	.278 ± .035*	.259 ± .024	.327 ± .076
udate	DA	12.1 ± 1.1	11.6 ± .5	11.3 ± .6	10.9 ± .8
	DOPAC	1.37 ± .11	1.35 ± .06	1.51 ± .15	1.36 ± .16
	DOPAC/DA	.114 ± .005	.118 ± .007	.132 ± .01	.122 ± .008
pothalamus	NE	2.19 ± .076	2.33 ± .138	2.2 ± .21	2.39 ± .111
	MHPG	.385 ± .013	.383 ± .02	.41 ± .018	.454 ± .013
	MHPG/NE	.177 ± .005	.167 ± .012	.2 ± .023	.192 ± .009
	DA	1.07 ± .086	.935 ± .075	.97 ± .125	1.03 ± .086
	DOPAC	.33 ± .024	.342 ± .02	.333 ± .027	.344 ± .023
	DOPAC/DA	.315 ± .021	.373 ± .021+	.357 ± .022	.342 ± .018
ain em	NE	1.08 ± .07	1.06 ± .06	1.03 ± .07	1.00 ± .05
	MHPG	.... ± .033	.252 ± .031	.266 ± .029	.269 ± .021
	MHPG/NE	.205 ± .019	.266 ± .018*	.285 ± .04	.252 ± .01

	DA	.13 ± .005	.127 ± .008	.124 ± .007	.126 ± .007
	DOPAC	.082 ± .004	.09 ± .005	.083 ± .007	.087 ± .004
	DOPAC DA	.634 ± .028	.716 ± .033^{+}	.67 ± .025	.7 ± .023
Spleen	NE	.868 ± .084	.938 ± .13	.77 ± .081	.915 ± .092

Mice were injected IP with Tf-5 and samples collected 60 min later. Amine analyses were by HPLC-EC.

$^{*}P < 0.05$, $^{+}$ $0.1 > P > 0.05$ relative to uninjected mice.

Table VI Plasma Corticosterone after PHA-stimulated Lymphocyte Supernatant or Thymosin Fraction 5

	Plasma Corticosterone (ng/ml)		
	Mean	SEM	%Vehicle
Vehicle	111 ±	18	[100]
Tf-5 (200 ug)	218 ±	41	196*
Tf-5 (800 ug)	134 ±	14	121
Cell supernatant	367 ±	40	331*

Mice were injected IP with spleen cell supernatants purified according to Besedovsky et al. (1983) or Tf-5 and plasma samples collected 2 hr later. The same experiment as Table V. Corticosterone concentrations were determined by radiomimmunoassay.

*$P < 0.05$ relative to vehicle-injected control.

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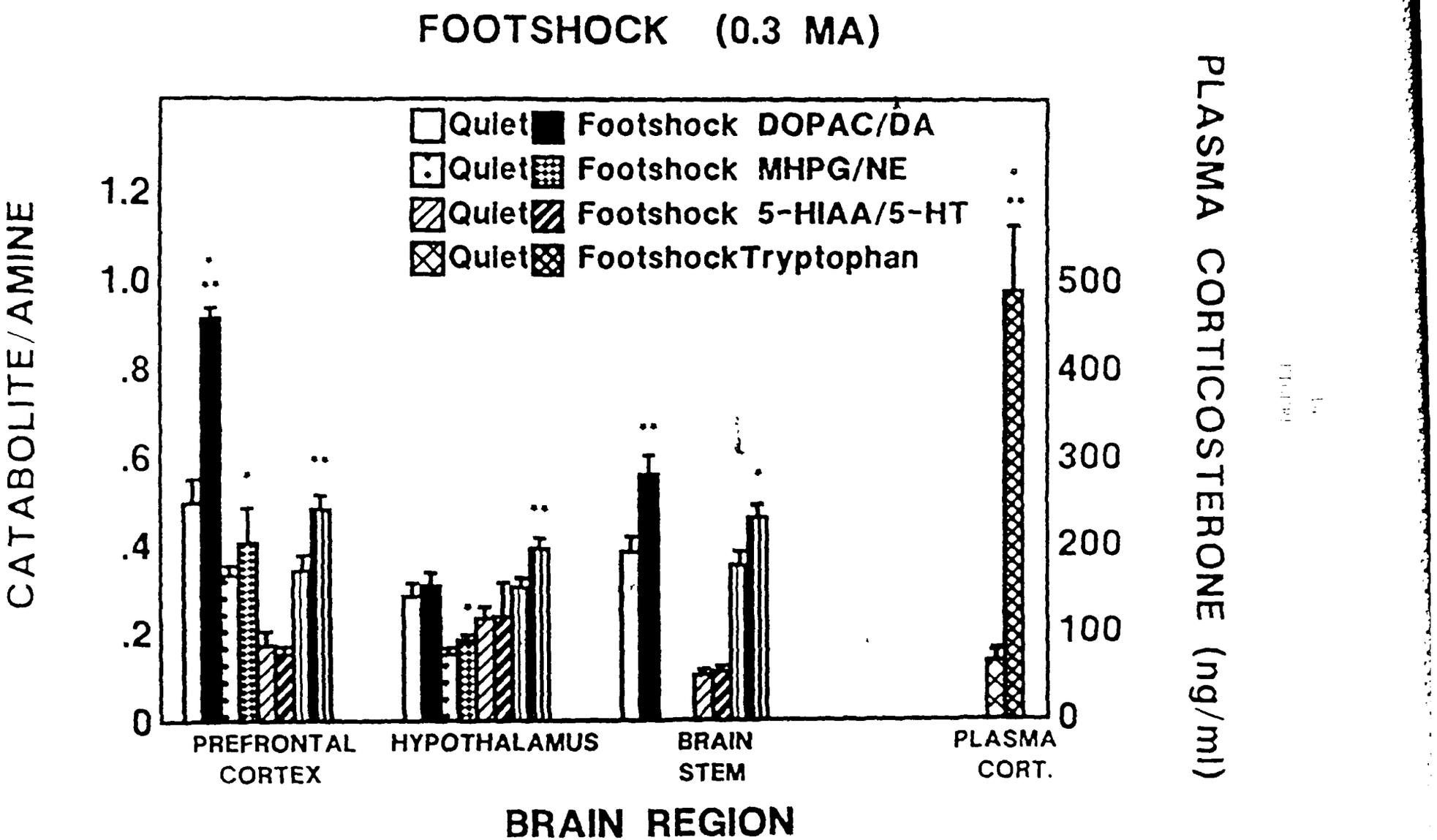
FOOTSHOCK (0.3 MA)
Quiet Footshock DOPAC/DA
Quiet Footshock MHPG/NE
Quiet Footshock 5-HIAA/5-HT
Quiet FootshockTryptophan
CATABOLITE/AMINE
PLASMA CORTICOSTERONE (ng/ml)
1.2
1.0
.8
.6
.4
.2
0
500
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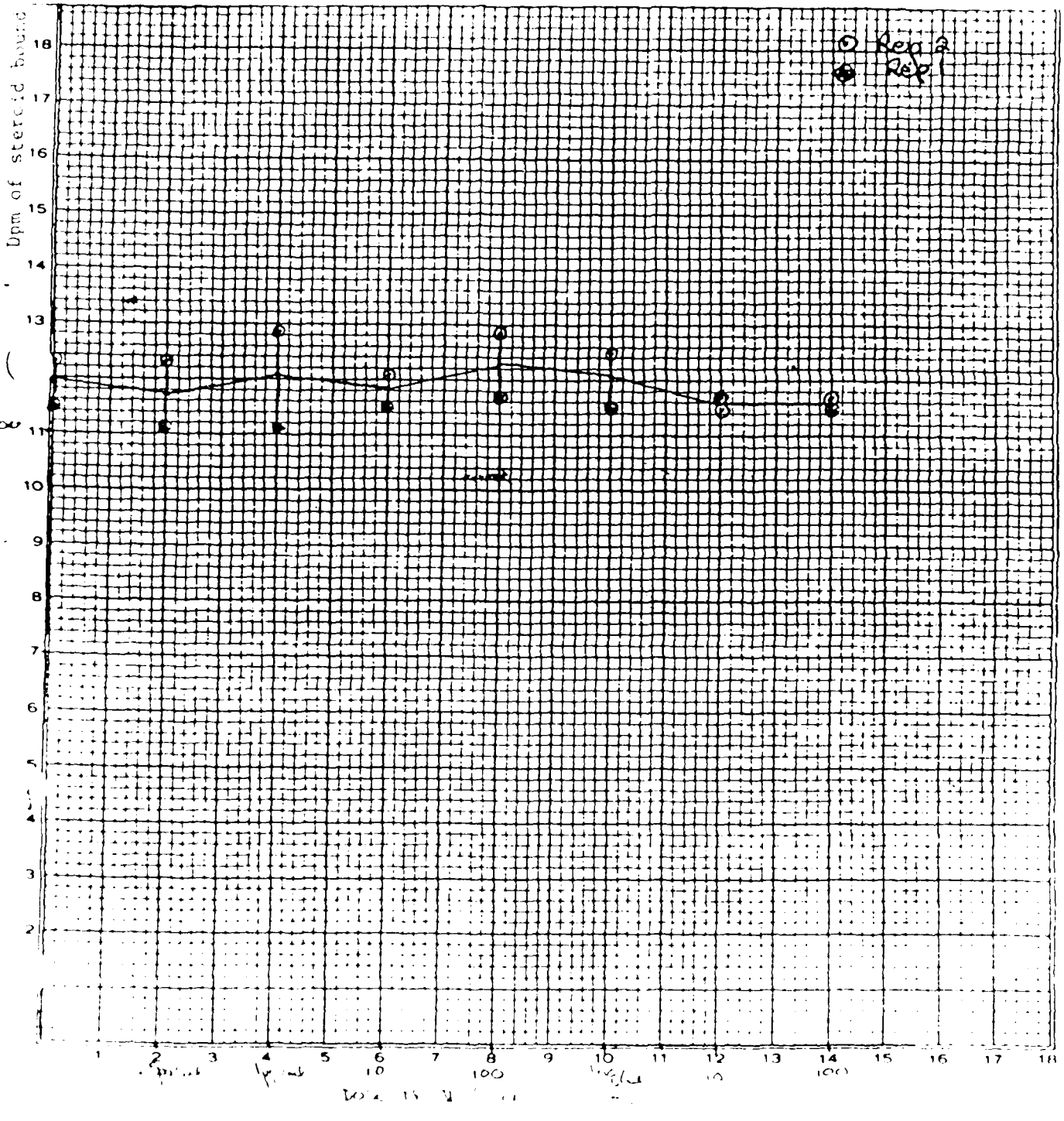
PREFRONTAL CORTEX
HYPOTHALAMUS
BRAIN STEM
PLASMA CORT.
BRAIN REGION

FIGURE 2



Concentration of Tf-5

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